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TRANSFORMATION OF AMINO-ACYL-s-RNA INTO DIPEPTIDYL-s-RNA BY MEANS OF A WATER-SOLUBLE CARBODIIMIDE

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ABSTRACT

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A study was carried out of the interaction between s-RNA from baker's yeasts and the acylating agent obtained from p-toluene sulphonate of N-cyclohexyl-N[β-(N-methyl-morpholinium)]-ethylcarbodiimide and from formylamino acids. It was shown that under the conditions used C^{14}-valyl-s-RNA is transformed into formylglycylvalyl-C^{14}-s-RNA with a yield of 70%.

Under similar conditions deacylated s-RNA combines with 7% C^{14}-formylalanine. No inactivation of s-RNA with regard to incorporation of C^{14}-valine by aminoacyl-s-RNA-synthetases from rat liver was noted.

A number of works have lately appeared which demonstrate that /189* an intermediate compound in protein biosynthesis is a polypeptide whose C-terminal amino-acid radical is linked by an ester bond to the 3'-hydroxy group of the terminal adenosine of the carrier ("soluble") ribonucleic acid (s-RNA)(Ref. 1 - 3). Throughout the growth of the

* Note: Numbers in the margin indicate pagination in the original foreign text

polypeptide chain, this intermediate compound is connected by its polynucleotide terminal to the ribosome. In a detailed investigation of the properties of these intermediate compounds and their behavior during protein biosynthesis, the possibility of deriving them artificially from molecules of the RNA carriers without using the ribosomal apparatus is of interest. One of the possible ways of solving this problem is to form a peptide bond between the corresponding amino acid or peptide, on the one hand, and the amino-acylated s-RNA, on the other hand.

Out of the numerous methods of synthesizing peptides for this purpose, the only suitable ones are those during which no chemical modification of the s-RNA molecule itself is produced, as a result of acylating the adenine and cytosine amino-groups of the ribose radical 2'-hydroxy groups. It is also desirable to conduct the reaction in an aqueous solution, since water is the most suitable solvent for s-RNA. Finally, the synthesis conditions must be mild enough to prevent separation of the amino-acyl radical from the s-RNA.

Laboratory work has recently shown that in an aqueous solution containing formylglycine and p-toluene sulphonate of N-cyclohexyl-N -[β-(N-methylmorpholinium)]-ethylcarbodiimide (CME-carbodiimide), a compound is formed which is capable of acylating the amino groups of amino-acid esters very rapidly at room temperature and pH 6-7 -(we shall give this compound the abbreviated name of acylating agent) (Ref. 4). The present work investigates the capacity of this acylating agent for reacting with the NH_2 group of the amino-acid radical of

aminoacyl-s-RNA, and also considers the problem of the degree of selectivity of this reaction.

METHODS

Formylglycine, formylalanine, and formyl-C^{14}-alanine are derived by Fischer's method (Ref. 5) from the corresponding amino-acids. The specific activity of the derived formyl-C^{14}-alanine is 180,000 imp/min.·μmole. Formylglycylvaline is derived by the previously described method (Ref. 6). The method described in Ref. 7 is used to derive the p.-toluene sulphonate of CME-carbodiimide.

The adenosine is a commercial preparation of the Reanal Company crystallized from water (Ref. 8) and dried over P_2O_5. The cytidine is a commercial preparation of the Calbiochem Company (United States). We produced the RNA carrier from baker's yeast by methods described for brewer's yeast (Ref. 9).

Aminoacyl-s-RNA-synthetases were made from rat liver (Ref. 10). /190 All operations took place at 2 - 4°. The liver, cut into tiny pieces by scissors, was homogenized for 1.5 min. in 0.05 M KCl (70 ml per the livers of nine rats). Then the homogenate was reduced to 180 ml of 0.05 M KCl and centrifuged for 1 hr. at 35,000 rpm in the No. 59 rotor of the VAC-40 ultracentrifuge. The suprasedimental fluid was brought by 1 M CH_3COOH to pH 5, and the precipitate was separated by centrifuging for 15 min. at 5,000 rpm. The precipitate obtained was suspended in a glass homogenizer in 30 ml of 0.1 M tris-HCl-buffer (pH 7.5), and the solution was centrifuged for 20 min. at 35,000 rpm in the VAC-40. The suprasedimental fluid was passed through a column

of diethyl-acetic-ether Sephadex A-25 (coarse), 0.6 x 5.0 cm, and balanced with 0.1 M tris-HCl-buffer of pH 7.5. The column was rinsed with three more milliliters of the same buffer. The eluate was poured into test tubes, refrigerated, and stored at -20°. Immediately before use, the enzyme solution was passed through a column of Sephadex G-50 (coarse), balanced with the same buffer. The columetric ratio of gel to enzyme solution was 10:1. Fractions of optical density not less than 10-12 at 280 mμ (10-12 mg of protein per milliliter) were used as the enzyme preparation.

In order to determine the capacity of s-RNA for enzymatically annexing amino-acid (acceptor activity), 0.5 mg of RNA were incubated with a preparation of aminoacyl-s-RNA-synthetase (1 mg of protein); 0.0015 μmole of C^{14}-valine (7500 imp/min.); 2.5 μmole of ATP; 2.5 μmole of Mg++; 0.25 μmole of ethylenediaminetetraacetic acid; and 12.5 μmole of tris-HCl-buffer, pH 7.5, in 0.5 ml of solution (Ref. 10). The reaction was stopped at certain time intervals by adding 0.4 ml of a 0.5%-solution of cetyltrimethylammonium bromide (cetavlone) in 0.6 M phosphate buffer, pH 6.2. The precipitate of the cetavlone salt of s-RNA was separated by centrifuging, and dissolved in 0.3 ml of 2 N NaCl. The RNA was precipitated with double the volume of ethyl alcohol. The NaCl precipitate of the s-RNA was dissolved in 0.3 ml of 2 N NaCl and again precipitated by double the volume of alcohol. The s-RNA precipitate was dissolved in 1 ml of water, and 0.5 ml of the solution was applied to the target to determine its radioactivity, while 0.2 ml was diluted with water to 3 ml to measure

its optical density at 260 mμ. These findings were used to compute the concentration of C^{14}-valine in the s-RNA in mμ moles of C^{14}-valine per 20 units of optical density of s-RNA (1 mg).

C^{14}-valyl-s-RNA was produced by incubating 20 mg of s-RNA in 20 ml of solution, having the same composition as was used when determining acceptor activity. The s-RNA precipitate obtained by precipitation with cetavlone after incubation was washed three times by alternate dissolution in 2 N NaCl and precipitation with alcohol. Then the s-RNA was dissolved in 12 ml of water, settled out with two volumes of alcohol with the addition of a 0.2 volume of 20% potassium acetate with pH 5.0, and the precipitate was dried over P_2O_5. The specific activity of the preparation obtained was 1600 imp/min. per 20 units of optical density.

The measurements of radioactivity were conducted on a BFL end-type radiation counter with a B2 radiometer.

The formation of the acylating agent of formylamino-acid and CME-carbodiimide, and the investigation of its interaction with nucleosides and s-RNA, was conducted in a cell with a loaded glass electrode connected by an electrolytic switch to a calomel electrode. The electrodes were connected to an LP-58 potentiometer. The pH was regulated during the reaction by slightly titrating the reaction mixture with a micropipette containing 1.0 N NaOH or 1 N H_2SO_4. The reaction proceeded at room temperature. A suspension of 0.2 μmole of CME-carbodiimide was added to the solution of formylamino-acid (0.2 μmole of formylglycine or 0.05 μmole of C^{14}-formylalanine) in

2 ml of water, titrated to pH 5.0; after 5 - 10 min. the pH changed to 6.5 - 7.0. A sampling was taken from the solution to determine the acylating agent concentration by the method previously described in Ref. 4. A nucleoside or s-RNA suspension was added to the remaining solution, the reaction was carried on for 30 min. while pH was kept between 6 - 7, and then the reaction mixture was analyzed for the acylation product.

Results of Investigation

The interaction of the acylating agent with adenosine and cytidine led, in principle, to the acylation of the ribose hydroxy groups and the amino-groups of bases. The capacity of esters of acylamino-acids and nucleosides to produce a hydroxamic reaction with a neutral solution of hydroxylamine was used to analyze the mixture for formylamino-acid ester content. This was determined by specific experiments on a sample of 2'(3'), 5'-di(carbobenzyloxyphenylalanyl) adenosine synthesized by the method in Ref. 11. In order to separate the reaction mixture from the acylating agent, which is also capable of producing a hydroxamic reaction, the reaction mixture was first passed through Dowex 50 x 12 in the form of triethylammonium. In the adenosine and cytidine experiments, the hydroxamic reaction with the eluates was negative, despite the substantial surplus of nucleosides as compared to the acylating agent (0.05 M of nucleoside per 0.003 - 0.004 M of /191 acylating agent). Therefore, under the conditions investigated no acylation was discovered with respect to ribose.

In order to determine the degree of acylation with respect to adenosine and cytidine amino-groups, the capacity of these nucleosides for being absorbed in Dowex 50 in H+ form was used. Thus, the quantity of N-aminoacylnucleosides may be judged from the optical density of the reaction mixture after passing it through a column of Dowex 50 (H+) and Dowex 1 (HCO_3^-) (to free it from the p-toluene sulphonate ion which is absorbed in the ultraviolet). The experiments showed that the optical density of the eluate in the case of both nucleosides was less than 1% of the optical density of the reaction mixture, and therefore that there was practically no acylation of the cytidine and adenosine amino-groups under the conditions under investigation.

These results led us to expect that aminoacyl-s-RNA acylation must proceed only with respect to the amino-group of the amino-acid radical. In order to ascertain definitively that no other parts of the s-RNA were subjected to acylation, we studied the interaction of the acylating agent with the s-RNA which contained no amino-acids. In these experiments, the acylating agent was derived from formyl-C^{14}-alanine. 15 mg of s-RNA ($\sim$ 0.6 μmole) were introduced into the reaction with about 2 μmole of acylating agent. After the reaction was over, the mixture was passed through columns with Dowex 50 x 12 [$(C_2H_5)_3NH^+$] and Dowex 1 x 8 (F^-) to free it from the acylating agent which had not completely reacted and from formylalanine; then the s-RNA was settled out by two volumes of alcohol. This precipitate was dissolved four times in water and settled out with alcohol (until there was no radioactivity in the suprasedimental fluid). Then the

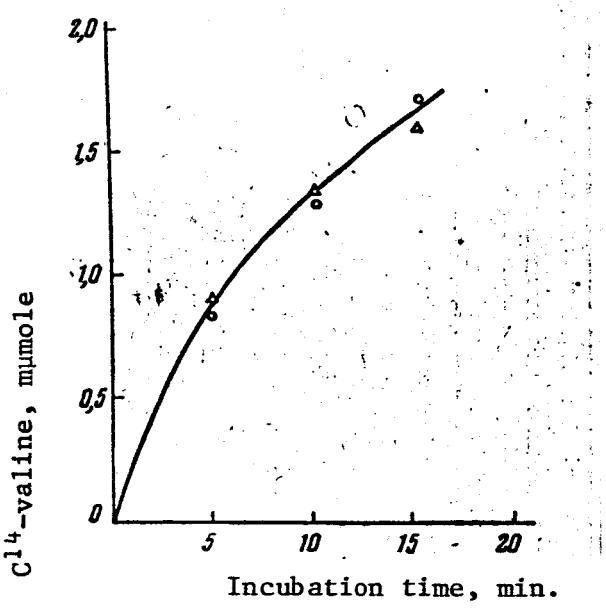

Figure 1

Kinetic Curves of C^{14}-valine Inclusion in Initial (Triangles) and s-RNA Treated with Acylation-Agent (Circles). Ordinate Axis - C^{14}-valine in mμmole per 20 Units of s-RNA Optical Density.

precipitate was dissolved, and its radioactivity and optical density were ascertained. Acylation of the unacylated RNA carrier with C^{14}-formylalanine gave the following results:

RNA, mg	Resultant imp/min/mg of s-RNA	C^{14}-formylalanine, μmole/μmole of s-RNA
15.0	320	0.075
14.8	280	0.065

Thus, in the absence of the aminoacyl radical, one molecule of formylalanine is annexed per 13 - 15 molecules of s-RNA, i.e., an insignificant degree of acylation occurs.

The acceptor activity of s-RNA was also examined, using the

example of the inclusion of C^{14}-valine after the unacylated s-RNA was treated with the acylating agent under investigation. Figure 1 /192 gives the kinetic curve of C^{14}-valine inclusion in s-RNA preincubated for 0.5 hr. at pH 6 - 7, with an acylating agent derived from formylalanine and CME-carbodiimide. It is evident that it is practically congruent with the kinetic curve for the original s-RNA which had not been exposed to the action of an acylating agent.

These experiments demonstrate that the acylating agent under investigation touches hardly any sectors of the s-RNA molecule which lack the aminiacyl radical, and does not affect the functional activity of s-RNA.

To study acylation with respect to the amino-group of the amino-acid radical bound to the s-RNA, 3 - 5 mg of C^{14}-valyl-s-RNA was introduced into a reaction mixture containing an acylating agent derived from formylglycine and CME-carbodiimide. After completion of the reaction, the s-RNA was settled out while cold by adding 0.3 ml of 1 N HCl. The precipitate was suspended in 1.5 ml of water, and again settled out with 0.15 ml of 1 N HCl. The precipitate was then dissolved in 0.01 N NaOH and kept at room temperature for 1 hr. Thus, both the non-acylated C^{14}-valine and the formylglycyl-C^{14}-valine which was formed should have been entirely eliminated. 5 mg of valine and 4 mg of formylglycylvaline were added to the solution as carrying agents, after which the solution was acidified to pH 5, and s-RNA was precipitated by adding two volumes of alcohol. The s-RNA precipitate contained no measurable amount of radioactivity. A portion of the suprasedimental fluid was passed through a column

of Dowex 50 x 12 (H^+), 1 x 15 cm, in order to separate the valine, and the radioactivity of the formylglycylvaline in the eluate was measured. Therefore, the valine was eluated from the column with 1 N ammonia, and the valine radioactivity was determined in the eluate. The ratio of the total radioactivities of both fractions directly gives the ratio of the acylated valine to that which did not enter into reaction. The Table gives the results of these two experiments together with those of two control experiments. In one of them, the C^{14}-valyl-s-RNA was introduced into a solution of CME-carbodiimide at pH 6, without first adding formylglycine, and in the second - into a mixture of 0.1 M formylglycine and 0.1 M CME-urea derived by catalytic hydration of CME-carbodiimide in an acid medium.

From these data it is apparent that in the presence of an acylating agent, 70% of the valine bonded with the s-RNA is acylated by formylglycine with the formation of dipeptide.

Some of the solution, obtained after hydrolysis of aminoacyl-s-RNA and dipeptidyl-s-RNA and after addition of the carrying agents, was subjected to paper chromatography in the (n) butyl alcohol-acetic acid-water system (4:1:1). The chromatogram was exposed in a Cl_2 /193 atmosphere, developed with a solution of 1% KI and 1% starch (Ref. 12), and cut into little strips to check the radioactivity in them (since the aim was qualitative identification, no correction was introduced for self-absorption by the paper). Figure 2 presents the results of the experiment. It is apparent that two principal radioactive peaks were obtained which coincide with the spots of valine and

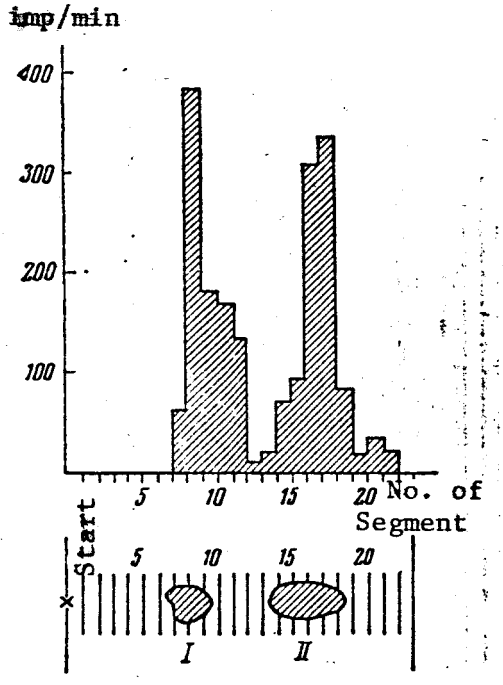

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Figure 2

Chromatogram Radioactivity Profile of C^{14}-valine and C^{14}-formylglycylvaline Derived by Separation from C^{14}-valyl-s-RNA after Treatment of the Latter by an Acylating Agent.

I - Valine; II - Formylglycylvaline.

formylglycylvaline on the chromatogram, i.e., practically all of /193 the radioactivity is contained precisely in these compounds.

Discussion of Results

Our research has shown that the unstable acylating agent formed in the interaction of CME-carbodiimide and formylamino-acids possesses a highly specific acylating action with respect to the aminoacylated RNA carrier. It gives a good yield in acylation of the amino-group

CONTENT OF C^{14}-VALINE AND C^{14}-FORMYLGLYCYLVALINE IN s-RNA AFTER INCUBATION OF C^{14}-VALYL-s-RNA WITH AN ACYLATING AGENT

Composition of reaction mixture into which C^{14}-valyl-s-RNA was introduced	Eluate radioactivity		Yield of acylation product, %
	aqueous	ammoniacal	
Formylglycine + CME-carbodiimide held for 5 min. at pH 5 (acylating agent)	3012	1267	70.4
The same	1186	520	69.9
CME-carbodiimide	----	1610	0
Formylglycine - CME-urea	----	1458	0

of the amino-acid radical, and hardly touches the remaining sectors of the molecule. The annexation which we found of one formylalanine radical per 13 - 15 molecules of s-RNA may be explained either by the insignificant quantity of amino-acids firmly bonded to s-RNA, or by reaction with any one of the rare bases contained in the s-RNA molecules. This matter requires specific study.

The specific acylation of the amino-group of the amino-acid radical, bonded to s-RNA, is described in Refs. 13 and 14, where N-carboxyanhydrides of amino acids were used for this purpose. The method described in those papers, however, makes it possible to derive only homopolymers of unregulatable chain length in a form bonded with s-RNA. The method which we are proposing makes it possible to produce peptidyl-s-RNA with a peptide of a prescribed

structure. In the present work, only dipeptidyl-s-RNA has been synthesized, but, as shown in our previous work (Ref. 15), CME-carbodiimide may be used to acylate amino-acid esters, not only with formylamino-acids, but also with formylpeptides. In the latter case, even higher yields are successfully obtained. Thus, the method may prove to be fairly universal.

Conclusions

It has been demonstrated that the unstable acylating agent formed in the interaction of p-toluene sulphonate of N-cyclohexyl-N -[β-(N-methylmorpholinium)]-ethylcarbodiimide with formylamino-acids is capable of acylating the aminoacyl radical bonded with carrier ribonucleic acid. Formylglycyl-C^{14}-valyl-s-RNA is obtained from C^{14}-valyl-s-RNA with a 70% yield.

It has been demonstrated that, under the conditions used for acylating the amino-group of the aminoacyl radical, neither acylation of ribose hydroxyls nor of cytosine and adenine amino-groups takes place, and there is practically no acylation of the s-RNA freed from the terminal amino-acid radical. It has been demonstrated that under these conditions no reduction in s-RNA acceptor activity occurs.

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